New diagnostic tools for different challenges in VL elimination

Malcolm S. Duthie
Senior Scientist
Elimination challenges

Disease → Cure/ Complication/Relapse
Elimination challenges

Disease  →  Cure/ Complication/Relapse

Detection
Surrogates – Ab, biomarkers
Direct – Ag, NAAT

Practicality....
Evaluation of diagnostic performance of rK28 ELISA using urine for diagnosis of visceral leishmaniasis

Prakash Giri1, Khondaker R. H. Bhunia2, Furat Hossain1, Md Arik Ashraf Khan1, Aarthy C. Valia3, Malcolm S. Dutta3, Shriram Haranath1, Md Abdul Salim1, M. Momen Huda1, Md Gulam Mozammel Khan1, Rhea N. Cole4, Sarven G. Reed5 and Dinsho Mondal1

Abstract
Background: Recombinant fusion proteins are now commonly used to detect circulating antibodies for the serodiagnosis of visceral leishmaniasis (VL) in Asia, Africa and the Americas. Although simple, these tests still require blood collection and their use in remote settings can be limited due to the need of collection devices, serum fractionation instrument and generation of biohazardous waste. The development of an accurate and non-invasive diagnostic algorithm for VL such as could be achieved with urine, is desirable.

Methods: We enrolled 87 VL patients and 81 non VL individuals, including 33 healthy endemic controls, 16 healthy non-endemic controls, 16 disease controls and 16 tuberculosis (TB) patients. We compared the efficacy of recombinant antigens rK28, rK39 and rKRM42 for the diagnosis of VL where either serum or urine were used to develop antibody detection ELISA.

Results: As expected, each of the antigens readily detected antibodies in the serum of VL patients. rK28 ELISA showed the highest sensitivity (98.85%), followed by rK39 and rKRM42 ELISA (97.7 and 94.44%, respectively), overall specificity was 96.7%. When urine was used as the test analyte, only a marginal drop in sensitivity was observed with rK28 ELISA again demonstrating the greatest sensitivity (95.44%). Followed by rK39 and rKRM42 ELISA, respectively. Again the overall specificity was 96.7%.

Conclusions: Our data indicate the potential for using urine in the diagnosis of VL. Detection of antibodies against rK28 demonstrated the greatest sensitivity. Together, our results indicate that rK28-based antibody detection tests using urine could provide a completely non-invasive tool amenable for diagnosis of VL in remote locations.

Keywords: Visceral leishmaniasis, Diagnosis, rK28, rK39, rKRM42, ELISA, Serum, Urine, Bangladesh

Table 4 Sensitivity and specificity of rK28 ELISA performed using serum and urine samples from VL patients and non-VL individuals for diagnosis of VL.

<table>
<thead>
<tr>
<th>Group</th>
<th>Subjects (n)</th>
<th>Positive (n)</th>
<th>Sensitivity (n, %)</th>
<th>Specificity (n, %)</th>
<th>95 % CI</th>
<th>Urine</th>
<th>Positive (n)</th>
<th>Sensitivity (n, %)</th>
<th>Specificity (n, %)</th>
<th>95 % CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>VL</td>
<td>87</td>
<td>86</td>
<td>86 (98.85)</td>
<td>na</td>
<td>93.76–99.97</td>
<td>83</td>
<td>83 (95.40)</td>
<td>na</td>
<td>na</td>
<td>88.64–98.73</td>
</tr>
<tr>
<td>All controls (EC + NEC + DC + TB)</td>
<td>81</td>
<td>3</td>
<td>na</td>
<td>78 (96.30)</td>
<td>89.56–99.23</td>
<td>3</td>
<td>na</td>
<td>78 (96.30)</td>
<td>na</td>
<td>89.56–99.23</td>
</tr>
<tr>
<td>EC</td>
<td>33</td>
<td>1</td>
<td>na</td>
<td>32 (96.97)</td>
<td>84.24–99.92</td>
<td>1</td>
<td>na</td>
<td>32 (96.97)</td>
<td>84.24–99.92</td>
<td></td>
</tr>
<tr>
<td>NEC</td>
<td>16</td>
<td>0</td>
<td>na</td>
<td>16 (100)</td>
<td>79.41–100</td>
<td>1</td>
<td>na</td>
<td>15 (93.75)</td>
<td>69.77–99.84</td>
<td></td>
</tr>
<tr>
<td>DC</td>
<td>16</td>
<td>1</td>
<td>na</td>
<td>15 (93.75)</td>
<td>69.77–99.84</td>
<td>1</td>
<td>na</td>
<td>15 (93.75)</td>
<td>69.77–99.84</td>
<td></td>
</tr>
<tr>
<td>TB</td>
<td>16</td>
<td>1</td>
<td>na</td>
<td>15 (93.75)</td>
<td>69.77–99.84</td>
<td>1</td>
<td>na</td>
<td>15 (93.75)</td>
<td>69.77–99.84</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: na, not applicable; 95 % CI, sensitivity or specificity at 95 % confidence interval.
Specific antibody responses as indicators of treatment efficacy for visceral leishmaniasis


Abstract Acute visceral leishmaniasis (VL) is caused by infection with parasites of the Leishmania donovani complex and may be fatal if not treated. Early diagnosis and efficacious treatment are the keys to effective VL management and control. Novel regimens are being developed to overcome limitations in VL treatment options, which are currently restricted by high costs, severe systemic side effects, and unresponsiveness. Although simple and accurate serological tests are available to help confirm VL, none are suitable to monitor treatment efficacy and cure. Here, we confirm that serum antibody responses to the diagnostic antigens rK39 and rK28 are unaltered by treatment, but demonstrate that antibodies produced against two antigens, rK26 and rK18, can be used as an indirect measure of parasite clearance. The levels of anti-rK18 and rK26 antibodies were high in patients at initial diagnosis but declined in patients treated with either SSG (Ethiopia) or Ambisome™ (Bangladesh). Taken together, we propose that serological tests which measure antibodies to rK26 and rK18 merit consideration as potential markers of treatment success and cure.
The Severity of Visceral Leishmaniasis Correlates with Elevated Levels of Serum IL-6, IL-27 and sCD14

Priscila L. dos Santos, Fabricio A. de Oliveira, Michelle Luiza B. Santos, Lucena Célina S. Cunha, Michelle T. B. Lino, Michelle F. B. de Oliveira, Manuela C. M. Brantini, Angela Maria Silva, Tatiana R. de Moura, Amélia R. de Jesus, Malcolm S. Duthie, Steven G. Reed, Roque P. de Almeida

1 Laboratório de Biologia Molecular, Hospital Universitário, Universidade Federal de Goiás, Goiânia, Brazil; 2 Programa de Pós Graduação em Ciências da Saúde, Universidade Federal de Goiás, Goiânia, Brazil; 3 Instituto de Investigação em Imunologia, São Paulo, Brazil; 4 Infectious Diseases Research Institute (IDRI), Seattle, Washington, United States of America

* nevipoachocamimida@gmail.com

Fig. 3. High IL-6 levels in serum is associated with disease severity. Serum levels of IL-6 and IL-27 measured before treatment by Luminex assay were compared in VL patients. (A) IL-6 levels in patients with classical VL (n = 25) and SVL (n = 12). (B) IL-6 levels in patients with classical VL (n = 25) and SVL that survived (n = 7) and SVL that died (n = 5). (C) IL-27 levels in patients with classical VL (n = 25) and SVL (n = 12).
RESEARCH ARTICLE

The Severity of Visceral Leishmaniasis Correlates with Elevated Levels of Serum IL-6, IL-27 and sCD14

Priscilla L. dos Santos¹, Fabrícia A. de Oliveira¹, Michel Luiza B. Santos¹, Luana Celina S. Cunha¹, Michelle T. B. Lino¹, Michelle F. S. de Oliveira¹, Manuela O. M. Bortolin¹, Angélica Maria Silva¹, Tatiana R. de Moura¹, Amélia R. de Jesus²*,³, Malcolm S. Doehle⁴, Steven G. Reed⁵, Roque P. de Almeida⁶,⁷*.

1. Laboratório de Biologia Molecular, Hospital Universitário, Universidade Federal de São Paulo, São Paulo, Brazil; 2. Programa de Pós-Graduação em Ciências da Saúde, Universidade Federal de São Paulo, São Paulo, Brazil; 3. Instituto de Investigação em Imunologia, São Paulo, Brazil; 4. Infectious Diseases Research Institute (IDRI), Seattle, Washington, United States of America

* rosapachecoalmeida@gmail.com

Fig 1. Serum levels of cytokines. Cytokines were measured by Luminox assay in sera of VL patients before (n = 25) and after treatment (n = 17) (D0 and D30, respectively), DTH+ (n = 11) and healthy control (n = 7). (A) IFN-γ, (B) IL-10, (C) IL-6, (D) IL-27 and (E) TNF-α.
Alteration of the serum biomarker profiles of visceral leishmaniasis during treatment

M. S. Duthie - J. Guerrier • A. Vallur • A. Bhatia • P. Lima dos Santos • E. Vieira de Melo • A. Ribeiro de Jesus • M. Troit • D. Montal
R. Almeida • S. G. Reed
Alteration of the serum biomarker profiles of visceral leishmaniasis during treatment

M. S. Duthe · J. Guadarian · A. Vallar · A. Bhatia · P. Lima dos Santos · E. Vieira de Melo · A. Ribeiro de Jesus · M. Teixeira · D. Mondal · R. Almeida · S. G. Reed
Development and comparative evaluation of two antigen detection tests for Visceral Leishmaniasis


Abstract

Background: Visceral leishmaniasis (VL) can be fatal without timely diagnosis and treatment. Treatment efficacy vary due to drug resistance, drug toxicity and comorbidities. It is important to monitor treatment response to ensure cure and to rule out relapse. Currently microscopy of spleen, bone marrow or lymph node biopsies is the only definitive method to evaluate cure. A less invasive test for treatment success is a high priority for VL management.

Methods: In this study, we describe the development of a capture ELISA based on detecting Leishmania donovani antigens in urine samples and comparison with the Leishmania Antigen ELISA, also developed for the same purpose. Both were developed as prototype kits and tested on patient urine samples from Sudan, Ethiopia, Bangladesh and Brazil, along with appropriate control samples from endemic and non-endemic regions. Sensitivity and specificity were assessed based on accurate detection of patients compared to control samples. One-Way ANOVA was used to assess the discrimination capacity of the tests and Cohen’s kappas were used to assess their correlation.

Results: The Leishmania Antigen Detect™ ELISA demonstrated 96% sensitivity on VL patient samples from Sudan, Bangladesh and Ethiopia and 88% from samples from Brazil. The Leishmania Antigen ELISA was comparable in performance except for lower sensitivity on Sudanese samples. Both were highly specific. To confirm utility in monitoring treatment, urine samples were collected from VL patients at days 0, 30 and 180 post-treatment. For the Leishmania Antigen Detect™ ELISA, positivity was highest at day 0 at 95% falling to 21% at day 30 and 11% at day 180, while all samples were negative, corresponding well with clinical cure. A similar trend was also seen for the Leishmania Antigen ELISA albeit with lower positivity of 91% at Day 0 and more patients remaining positive at Days 30 and 180.

Discussion: The Leishmania Antigen Detect™ and the Leishmania Antigen ELISAs are standardized, user-friendly, quantitative and direct tests to detect Leishmania during VL, as well as to monitor parasite clearance during treatment. They are a clear improvement over existing options.

Conclusion: The ELISAs provide an non-invasive method to detect parasite antigens during acute infection and monitor parasite clearance, allowing an unmet need in VL management. Further refinement of the tests with more samples from endemic regions will delineate their utility in monitoring treatment.

Keywords: Diagnosis, Leishmania, Antigen, Treatment, Antibody, Spleen, Infection
Elimination challenges

Disease → Cure/ Complication/Relapse

Infection →

Evaluation *en masse*
(practical, objective)

Intervention
(short-term, repetitive *versus* long-term, sustainable)
Accurate Serodetection of Asymptomatic *Leishmania donovani* Infection by Use of Defined Antigens

Aarthi C. Valluru, Caroline Reinhart, Raodoh Mohamath, Yasuyuki Goto, Prakash Ghosh, Dinash Mondal, Malcolm S. Duthie, Steven G. Reed

Infectious Disease Research Institute, Seattle, Washington, USA; International Center for Diarrheal Disease Research, Laboratory Sciences Division, Dhaka, Bangladesh

Infection with *Leishmania donovani* is typically asymptomatic, but a significant number of individuals may progress to visceral leishmaniasis (VL), a deadly disease that threatens 200 million people in areas where it is endemic. While diagnosis of acute VL has been simplified by the use of cost-effective confirmatory serological tests, similar standardized tools are not widely available for detecting asymptomatic infection, which can be 4 to 20 times more prevalent than active disease. A simple and accurate serological test that is capable of detecting asymptomatic *L. donovani* infection will be useful for surveillance programs targeting VL control and elimination. To address this unmet need, we evaluated recombinant antigens for their ability to detect serum antibodies in 104 asymptomatic *L. donovani*-infected individuals (qualified as positive for *L. donovani*-specific antibodies by direct agglutination test (DAT)) from the Mymensingh district of Bangladesh where VL is hyperendemic. The novel protein rTR18 and rTR18B possessed the greatest potential and detected 69% of DAT-positive individuals, with rTR18 being more robust in reactivity. Agreement in results for individuals with high DAT responses, who are more likely to progress to VL disease, was 74%.

When considered along with rK90, a gold standard antigen that is used to confirm clinical diagnosis of VL but that is now becoming widely used for surveillance, rK90 and rTR18 conferred a sensitivity of 84% based on a theoretical combined estimate. Our data indicate that incorporating rK90 and rTR18 with rK90 in serological tests amenable to rapid or high-throughput screening may enable simple and accurate detection of asymptomatic infection. Such tests will be important tools to measure *L. donovani* infection rates, a primary goal in surveillance and a critical measurement with which to assess elimination programs.

**FIG 1** Assessment of the ability of a serological test comprising recombinant proteins to detect antibodies in the serum of asymptomatic infected individuals. Asymptomatic infected individuals were defined as DAT positive, and serum antibodies against the indicated antigens were measured by ELISA. The optical density of each serum sample from asymptomatic individuals (Asympt) (circles), VL patients (VL) (squares), healthy non-endemic controls (NEC) (triangles), DAT-negative healthy endemic controls (EC) (inverted triangles), and controls with other diseases (OD) (diamonds) is plotted. Median optical density is indicated for each group (black bars). The black line intersecting each plot identifies the cutoff above which samples were considered positive. ****, P value of <0.0001; ***, P value of <0.005 and <0.01, respectively, as measured by one way ANOVA.
Asymptomatic infected individuals

Direct Agglutination Test (DAT)

Lysate ELISA

rK39 ELISA
Asymptomatic infected individuals

![Graphs showing Ld DNA equivalents/mL over time for PCR negative and PCR positive at 12 month follow-up.](image)
Progress to clinical study (F3+GLA-SE)

From mouse to man: safety, immunogenicity and efficacy of a candidate leishmaniasis vaccine LEISH-F3+GLA-SE

Rhea N Coler1, Malcolm S Duthie1, Kimberly A Hofmeyr1, Leelaheni Jayashankar1, Julie Vergnet2, Toen Rollf2, Ayesha Masgith3, John D Lawrence3, Varasha S Roman1, H Henry Ballou4, Naradha Dabola Causerart5, Steven J Reed1, Anshu Vahli1, Michelle Favila5, Mark T Orr1, Jill Ashman1, Pralosh Gohsh6, Dinesh Mondal2 and Steven G Reed1

Key antigens of Leishmania species identified in the context of host responses in Leishmania-exposed individuals from disease-endemic areas were prioritized for the development of a subunit vaccine against visceral leishmaniasis (VL), the most deadly form of leishmaniasis. Two Leishmania proteins — a Nuclear Hsp70 and a stenol 24-kDa-thyroid antigen, each of which are protective in animal models of VL when properly adjuvanted — were produced as a single recombinant fusion protein (F3-GLA-SE) for use as antigen production and broad coverage of a heterogeneous major histocompatibility complex population. When formulated with glycosylated liposome A-stable adjuvant water emulsion (GLA-SE), a Toll-4-like vaccine d TPM (T-helper 1) promoting nonamplification adjuvant, the LEISH-F3 polypeptide induced potent protection against both L. donovani and L. infantum in mice, measured as significant reductions in liver parasite burdens. A robust immune response to each component of the vaccine with polyfunctional CD4+ T-cell responses characterized by production of antigen-specific interferon-γ, tumor necrosis factor and interleukin-2 (IL-2) and low levels of IL-6 and IL-10 was induced in immunized mice. We also demonstrate that CD4 T cells, but not CD8 T cells, are sufficient for protection against L. donovani infection in immunized mice. Based on the sum of preclinical data, we prepared GMP materials and performed a phase 1 clinical study with LEISH-F3-GLA-SE in healthy, uninfected adults in the United States. The vaccine candidate was shown to be safe and induced a strong antigen-specific immune response, as evidenced by cytokine and immunoglobulin subclass data. These data provide a strong rationale for additional trials in Leishmania-endemic countries in populations vulnerable to VL.
Immunization with NSΔC (F3+) reduces *Leishmania* infection. Mice were subcutaneously injected a total of 3 times with 5ug protein formulated with GLA-SE, then one month after the final immunization were infected by intravenous injection of *Leishmania* promastigotes. Livers were removed one month after inoculation and parasite burdens were determined by qPCR. In (A) and (B), C57BL/6 and BALB/c mice, respectively, were infected with *L. donovani*. In the lower panels, C57BL/6 and BALB/c mice were infected with *L. infantum*. Each point represents the burden of each individual mouse, with the bars indicating the mean and SE for each group with 7-10 mice per group. Data are representative of results obtained with each protein in 2-3 independent experiments.

**CONCLUSION**

F3+ candidate is highly and robustly protective against *Leishmania* infection
Immunization with NSAC reduces parasite burden in hamsters infected with *L. infantum* during sandfly bloodmeals.

**CONCLUSIONS –**

a. F3+ candidate provides more robust, statistically significant reduction in parasite burden.

b. GLA-SE adjuvanted F3+ promotes protective response.
Elimination challenges

rK39 RDT →
(biomarkers/ Ag/ Ag/ PCR)

Ab/ Ag/ PCR →
Screening – high-throughput lab based/ portable, field-applicable

Intervention – IRS, vaccine